PHYSIOLOGICAL SALT SOLUTION FOR THE LAND CRAB, GECARCINUS LATERALIS

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The land crab, *Gecarcinus lateralis,* is an active responsive animal until it approaches ecdysis; in the few weeks before and after ecdysis, however, the animal becomes lethargic (Bliss, 1962). These variations in activity may well be related to the marked changes in the metabolism of somatic muscle during molting (Skinner, 1962; 1963a, 1963b). In order to investigate the physiology of this muscle it has first been necessary to develop a Ringer's solution appropriate to the animal. This paper describes analyses performed on *Gecarcinus* serum. Observations on osmoregulation are included. From these data a Ringer's solution has been devised and tested.

Materials and Methods

1. *Animals*

Some specimens of *Gecarcinus lateralis* were collected in the field at Bimini and were used immediately at the Lerner Marine Laboratory. Other specimens were shipped from the Bermuda Biological Station to New York and were housed in covered aquaria containing sand moistened with tap water. A fingerbowl of sea water was available in each tank; where indicated, tap water was substituted for sea water.

2. *Preparation of blood serum*

Blood was collected from the cut appendage of an animal which had been acutely chilled to prevent autotomy of the cut limb. The clot was mechanically disrupted and sedimented by centrifugation.

3. *Osmolality, sodium, potassium and chloride*

Immediately after preparation, the osmolality of the serum was measured in a Fiske osmometer. Concentrations of sodium and potassium in the serum were determined by standard flame-photometric methods, using LiCl as an internal standard.

Chloride was determined by the Cotlove titrimetric method (Cotlove *et al.*, 1958). Initial attempts to determine chloride concentration of untreated blood serum led to results varying by as much as 15% on five replicate samples. Since the protein concentration of *Gecarcinus* serum is high and variable (2% to 10%, unpublished data), we thought that protein might be interfering with the analyses.

Consequently, the protein precipitated by the nitric-acetic acid reagent used in the analysis was homogenized to free any trapped chloride and removed by centrifugation. Aliquots of the supernatant were used for the titration. This procedure reduced the variability between replicate samples to less than 2%.

4. *Calcium and magnesium*

(a) *Preparation and characterization of an ultrafiltrate.* Blood was ultrafiltered to obtain a value for free calcium and magnesium without including divalent ions associated with proteins. Three-inch dialyzer tubing (average pore diameter 48 Å) was cut along its edge, giving a piece 6 inches wide. This was shaped into a sack and inserted into the top of a 12-ml. conical centrifuge tube. One to 2 ml. of blood were introduced into the sack which was then tightly stoppered and centrifuged. The first fluid collected after bringing the centrifuge to speed was set aside as possible condensate from the tubing. TCA was added to each of these initial collections. In the rare event that any precipitate formed (indicating the presence of protein and hence a leak in the system), the sample was transferred to another dialysis sack. The tubes were then spun at 3000 rpm in a model CM International centrifuge for two hours. Heating was prevented by packing the drive shaft of the centrifuge in dry ice during the centrifugation. The rate of ultrafiltration was about 0.025 ml. $hr.^{-1}$ ml. $serum^{-1}$. The ultrafiltrate obtained was colorless and contained no more than .06% protein (as determined by the method of Lowry *et al.*, 1951), representing about 1% of the protein initially present in the serum. Within the limits of experimental error, the alkali metal concentrations in the ultrafiltrate were the same as those in whole serum. Since the small correction for serum water would be opposite to that applied for the Donnan equilibrium, the similarity was an expected result, and indicated that there was no significant evaporation of the ultrafiltrate during preparation.

(b) *Assay method.* The dye Eriochrome Black T is pink when chelated to divalent cations and blue when free in solution after the cations have been removed by a stronger chelating agent. With this dye as an indicator, the sum of calcium and magnesium was titrated with EDTA at a basic pH in the presence of cyanide (Ames and Nesbett, 1958). Calcium alone was determined titrimetrically on separate aliquots of each sample, using 2-hydroxy-1-(2-hydroxy-4-sulfo-1-naphthyl-azo)-3-naphthoric acid (HHSNN, Fisher Scientific Company) as indicator and EGTA (ethylene glycol bis (β-aminoethyl ether)-N, N-tetraacetic acid) as the titrant (Weber and Herz, 1963). Magnesium was obtained by subtracting the calcium value from the total. Standard curves were run with each set of experimental samples.

5. *Sulfate*

Proteins were precipitated from serum with perchloric acid (0.7 *M* final concentration). The supernatant was neutralized with KOH and the concentration of inorganic sulfate measured according to the method of Jones and Letham (1956). In four experiments, where known amounts of sulfate were added to crab serum, 101.5% of the added sulfate was recovered.

6. *pH, pCO_2, pO_2, and bicarbonate*

Blood was collected by immersing a cut appendage below the surface of paraffin oil saturated with water. The clot was mechanically disrupted and the serum transferred anaerobically to a cuvette housing a Clark polarographic O_2 electrode, a Severinghaus CO_2 electrode, and a glass pH electrode, all of which were read out by means of a Beckman model 160 gas analyzer. The pH of any sample which differed significantly from the others was measured independently with a Radio-

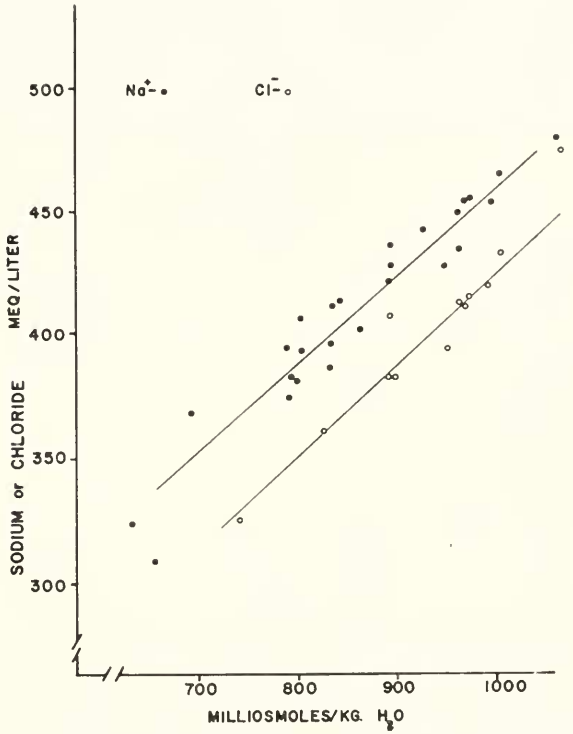

FIGURE 1. Sodium and chloride as functions of osmolality in the serum of *Gecarcinus lateralis*. *Sodium data:* closed circles; *chloride data:* open circles. Regression lines were fitted to the data by the method of least squares.

meter pH meter; in such cases the two readings always checked within 0.02 pH unit. In addition pH measurements were made after equilibration of samples with varying concentrations of CO_2 in air, in order to obtain the apparent pK. With this pK and the measured pH and pCO_2, the bicarbonate concentrations were calculated.

7. *Inorganic phosphate*

Protein was precipitated from serum with TCA (trichloroacetic acid) at a final concentration of 5%. Inorganic phosphate was determined by the method of Fiske and SubbaRow (1925).

Results and Discussion

1. *Osmolality, sodium, potassium and chloride*

The osmolality of *Gecarcinus* serum varied from 610 to 1060 mosm/kg. H_2O, depending on environmental conditions. Sodium varied from 310 to 480 meq/L. and, in any given animal, accounted for approximately one-half the total osmolality (Fig. 1). Chloride, the principal serum anion, was also linearly related to the osmolality but was present at concentrations about 35 meq/L. less than the sodium in the 12 sera analyzed for both ions.

Serum potassium varied from 7 to 15 meq/L. Figure 2 shows that the potassium concentration also tended to vary with osmolality, but in this case the data were proportionately more scattered and the interdependence was not as evident until we obtained the data on osmoregulation described below.

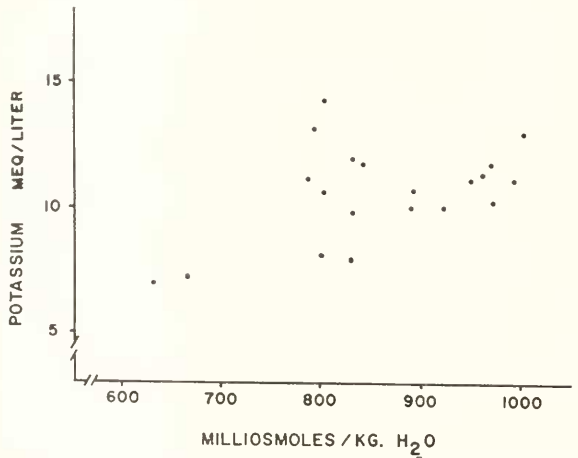

Figure 2. Potassium as a function of osmolality in the serum of *Gecarcinus lateralis.*

During the initial phases of this work at Bimini, 21 animals sampled immediately after they were caught in the field had an average serum sodium of 369 ± 28 (S.D.) meq/L., while 8 animals kept on moistened sand (but with no other source of water) for 48 hours before sampling had an average serum sodium of 456 ± 26.3 (S.D.) meq/L. This difference was highly significant and prompted further investigation of the effects of environmental conditions.

In Bimini, *Gecarcinus* burrows in the sand some distance from the sea in an area where the ground water is salty. Animals shipped to New York have, shortly after arrival, a serum osmolality of approximately 830 mosm/kg. H_2O. A group of animals was kept from arrival in an aquarium with sea water [1] available in the water bowls; after several weeks a blood sample was taken for osmolality, sodium, potassium and chloride determinations. The animals were replaced in tanks

[1] The sea water used was obtained from the New York Aquarium (Coney Island) and had the following measured composition (in meq/L.): Sodium, 375; potassium, 8.1; chloride, 460; magnesium, 79.0; calcium, 16.4; osmolality = 860 mosm/kg. H_2O; salinity = 27.1‰.

with tap water in the bowls, and after 8 days (three animals) or 27 days (two animals), the blood collections and determinations were repeated. The results (Table I) show that all four parameters decreased when only fresh water was available, and that the decrease was greater after the longer exposure. These experiments show that potassium, as well as sodium and chloride, does vary in the same sense as the osmolality, a relationship not readily seen from the data in Figure 2.

2. *Calcium and magnesium*

The mean value for free calcium in serum water of intermolt animals was 17.2 ± 2.4 (S.D.) meq/L.; the mean value for free magnesium was 13.8 ± 2.2 (S.D.) meq/L. (Table II).

TABLE I

Osmolality, sodium, potassium and chloride of Gecarcinus serum in animals given access to sea water only for several weeks (I) and thereafter given access to tap water only (II)

Animal	Days of access to tap water	Osmolality (mosm/kg. H_2O)		Sodium (meq./L.)	Chloride (meq./L.)	Potassium (meq./L.)
1	8	I	1000	467	435	13.0
		II	890	423	385	10.8
2	8	I	965	456	414	11.7
		II	890	438	410	10.5
3	8	I	990	456	422	11.1
		II	960	452	415	11.3
4	27	I	948	430	396	11.2
		II	825	388	362	8.0
5	27	I	1058	457	418	11.5
		II	895	430	385	10.0

These values are distinctly lower than those found by Gross (1963) for *Gecarcinus*. The difference probably reflects the fact that Gross dialyzed the serum against distilled water (for his method, see Gross, 1959), a procedure which would be expected to release cations normally bound to protein.

We found that two premolt animals had calcium concentrations higher than the average intermolt level, whereas the magnesium levels were the same at both stages (Table II). After ecdysis, the calcium level fell while the magnesium level rose more than 40%. Travis (1955) has described a similar pattern for calcium in the pre- and postmolt periods for another crustacean, the spiny lobster. Although these changes are of interest in the overall electrolyte metabolism of molting, we considered them too small to influence significantly the physiological effectiveness of a Ringer's solution; therefore we did not sample a larger series of pre- and postmolt animals.

TABLE II

Concentration of calcium and magnesium in Gecarcinus serum ultrafiltrate

Stage	Animal	Calcium (meq./L.)	Magnesium (meq./L.)
Intermolt	1	13.2	15.2
	2	18.5	11.5
	3	17.4	15.7
	4	24.8	10.0
	5	18.4	17.6
	6	17.1	15.3
	7	19.2	13.2
	8	17.6	12.8
	9	16.8	12.4
	10	16.8	14.4
	11	14.3	
	12	13.5	Avg: 13.8 ± 2.2 (S.D.)
		Avg: 17.2 ± 2.4 (S.D.)	
Premolt	1	22.0	14.0
	2	24.8	13.6
		Avg: 23.4	Avg: 13.8
Postmolt	1	18.4	17.6
	2	20.0	18.0
	3	18.8	23.2
		Avg: 19.1	Avg: 19.6

3. *Inorganic sulfate*

The results of 17 analyses are listed in Table III. Sera from 13 intermolt animals had an inorganic sulfate concentration of 11.18 ± 0.66 (S.D.) meq/L. Two premolt and two postmolt animals had similar values, indicating no variation during the molt cycle.

4. *pH, pCO_2, pO_2 and bicarbonate*

Gecarcinus blood serum has a relatively constant pH of 7.2 and a pCO_2 of 14 mm. Hg (Table IV). The wide fluctuations observed in the oxygen tension are unexplained. They are probably not due to the mixing of "arterial" with "venous"

TABLE III

Inorganic sulfate in Gecarcinus serum

Stage	Number of animals	Inorganic sulfate	
		Range (meq./L.)	Average (meq./L.)
Intermolt	13	10.20–11.94	11.18 ± 0.66 (S.D.)
Premolt	2	10.86–11.06	10.96
Postmolt	2	10.92–11.68	11.30

TABLE IV

pH, pCO_2, pO_2, bicarbonate in Gecarcinus serum

Animal	pH	pCO_2 (mm. Hg)	pO_2 (mm. Hg)	Bicarbonate (meq./L.)
1	7.20	14	24	7.40
2	7.22	13	29	5.14
3	7.43	12	28	7.70
4	7.14	12	72	3.95
5	7.05	16	56	4.15
6	7.08	15	36	4.99
7	7.26	16	30	6.94
8	6.95	14	46	2.97
Averages	7.17	14	40	5.40

blood (if such terms can be used to describe the hemolymph of an arthropod), since if mixing were the cause of the variability, we would expect low pO_2 values to be correlated with high pCO_2 values.

The pCO_2 is considerably higher than that of the sera of many other invertebrates (Spector, 1956; p. 270). The low pCO_2 of insects is probably due to the direct oxygenation of every cell by tracheole penetration, while the low pCO_2 of various marine Crustacea is probably due to the solubility of CO_2 in the sea water bathing the gills.

To determine the site of the diffusion barrier for CO_2 in *Gecarcinus,* the branchial chamber of an animal was flushed with 100% O_2 for 10 minutes before and throughout the collection of the blood sample. The pO_2 of that serum was only 52 mm. Hg, while the pCO_2 was 13.5 mm. Hg. The maintenance of this high pCO_2 in the blood despite the fact that the gill chamber was flushed free of CO_2 indicates that the barrier lies between the gill chamber and the branchial chamber. Further experiments will be performed to test this possibility.

5. *Inorganic phosphate*

The inorganic phosphate content of 31 serum samples collected from animals in the field averaged 0.76 but ranged from 0.21 to as high as 2.08 mmols/liter (Table V).

TABLE V

Inorganic phosphate in Gecarcinus serum

Stage	Conditions	Number of animals	Inorganic phosphate range (mmoles/L.)	Average
Intermolt	Collected in field	31	0.21–2.08	0.76
Intermolt	Starved >3 days	7	0.29–0.53	0.42
Premolt ($D_0 - D_4$)	Did not eat	8	0.33–0.78	0.50
Day of ecdysis	after onset	1	0.42	0.42
Postmolt ($A - C_1$)	of D_0	12	0.34–0.66	0.51

Travis (1955) found that under controlled feeding conditions the level of inorganic phosphate in the serum of the spiny lobster remained relatively constant throughout the molt cycle. A decrease of 25% in the postmolt period was the greatest fluctuation she observed. According to Travis, diet was the principal factor which determined serum phosphate levels.

We took our blood samples in the field within a few hours after the animals had been collected; therefore, the time and content of each specimen's most recent meal probably accounted for the 10-fold variation in inorganic phosphate level. More recently we have analyzed blood from a group of animals maintained in the laboratory, where feeding conditions could be controlled. Sera from these animals starved for three or more days showed much less variation in the inorganic phosphate concentration and were in the lower range of those collected in the field (Table V).

There was no correlation of inorganic phosphate concentration with the molting cycle. These data appear to be similar to those obtained by Travis for the spiny lobster.

TABLE VI

Composition of Ringer's solution for Gecarcinus

Compound	mmols./L.
NaCl	430
K_2SO_4	5
$MgCl_2$	7
$CaCl_2$	9
"Tris" buffer	10

The final pH is adjusted to 7.2 with 0.2 N maleic acid.

6. *Preparation and physiological efficacy of the Ringer's solution*

Based on the measurements above a Ringer's solution has been devised (Table VI).[2] Regardless of the serum osmolalities (and corresponding ion concentrations) within the range of 610 to 1060 mosm/kg. H_2O, there were no gross behavioral differences in the specimens of *Gecarcinus*. Hence it appeared that a Ringer's solution within this range should support normal neuromuscular activities. We have selected a sodium concentration (430 meq/L.) and osmolality close to the values observed in animals shortly after their arrival in the laboratory. The use of chloride salts raised the concentration of chloride somewhat higher than any observed in the animals. Since the final Ringer supported prolonged neuromuscular activity, the high chloride does not appear to exert any deleterious effect.

[2] Previous data published on the ionic composition of *Gecarcinus lateralis* serum by Prosser and Brown (1962; p. 60) were preliminary data obtained by J. W. Green in collaboration with one of us (DMS). Since for technical reasons we were not confident of the validity of some of the numbers obtained at the time, we did not publish the data. However, we did make them available to a few colleagues, one of whom submitted them for publication to Dr. Prosser.

Dr. Prosser published them in good faith without knowledge of their source. Before undertaking the present work, we tested a Ringer's solution prepared from the values obtained earlier and found that it did not support nerve or muscle function.

The solution is brought to pH 7.2 with Tris(hydroxymethyl) aminomethane/maleic acid, a buffer commonly used in crustacean Ringer's solution (Elliott and Florey, 1956). The concentration of inorganic phosphate in *Gecarcinus* plasma was too low to use it as an effective buffer. Indeed, the small concentration prompted us to omit inorganic phosphate from the solution entirely. We have also omitted bicarbonate from the Ringer since its buffering capacity at 5×10^{-3} *M* would be small and would require in any case the maintenance of a constant pCO_2.

The measured osmolality of the final solution was 850 mosm/kg. H_2O.

A chela of an intermolt animal was removed and the nerve trunk in the merus was freed of all surrounding tissue. Forty ml. of the Ringer were perfused through the cut end of the propus to wash out blood. The nerve trunk was stimulated and the contraction of the adductor muscles in the propus was observed intermittently over a four-hour period. During the same period of time, sensory stimulation (*i.e.,* light taps) in the region of the mechanoreceptors in the leg joints elicited action potentials which could be recorded approximately 4 cm. down the sensory nerve. Thus, axonal conduction, neuromuscular transmission, and muscular contraction appear normal for up to four hours, at which time the experiments were terminated.

Conclusions

Summing the inorganic ions for an animal with an osmolality in the median range, *e.g.,* 850 mosm/kg. H_2O, we find that the inorganic cations total about 450 meq/L. and the inorganic anions about 385 meq/L., leaving 65 meq/L. anionic charge unaccounted for. Acidic amino acids contribute little to this charge since they are present in low concentrations (*ca.* 0.05–0.10 mmol/liter total) and are more than balanced by basic amino acids (*ca.* 0.13–0.74 mmol/liter, unpublished data). The protein concentration in *Gecarcinus* serum is high and the isoelectric points of all the proteins are not known. Most should be negatively charged at the pH of the animal's plasma unless the isoelectric points are unusually basic. Therefore negative charges on protein probably account for many of the undetermined anions.

In a serum of osmolality 850 mosm/kg. H_2O the total of all inorganic ions is about 815 mmol/L. If we assume a rational osmotic coefficient of about 0.9 for these electrolytes, only 13% of the total osmotic pressure is unaccounted for. Much of this will be made up by proteins, amino acids, glucose, and other commonly occurring organic solutes. Therefore it is unlikely that any single organic compound makes up an important fraction of the total osmotic pressure, as does urea in elasmobranchs (Prosser and Brown, 1962; p. 142) and glycerol in some insects (Wyatt and Meyer, 1959).

The clear dependence of sodium, chloride and potassium concentrations and of osmolality on the nature of the available water supply indicates that *Gecarcinus* does not regulate these concentrations about a critical set point. A corollary of this conclusion is the observation that all animals showed similar motor behavior regardless of their plasma ion concentrations. It is of interest that the serum osmolality is always greater than that of the available water supply. *Gecarcinus* is a land animal and evaporation at its gills undoubtedly leads to the observed hemoconcentration. Gross' data (1963) show that concentrations of alkali metal cations are essentially the same in urine as in blood over a wide range of blood concentrations. This

observation precludes the possibility that renal mechanisms compensate blood changes.

Gross measured sodium and potassium in groups of *Gecarcinus* exposed to a variety of environments; he concluded, as have we, that the concentrations of these two ions are not closely regulated. Flemister (1958) immersed the animals in aqueous solutions of various chloride concentrations and found that even after several days the blood chloride did not equal environmental chloride. Under these conditions Flemister noted that animals immersed in hypotonic sea water had blood chloride concentrations greater than that of the environment, whereas animals immersed in hypertonic sea water had blood chloride less than that of the environment. In his experimental situation the normal evaporative processes are prevented. However, his data indicate, as do ours, that the plasma chloride concentration decreases in a hypotonic medium and increases in a hypertonic environment.

Whether one concludes that *Gecarcinus* is capable of osmoregulation depends to some extent on one's definition of the term. Serum ion and osmolality levels are not maintained constant independent of the environment; but not even in the case of total immersion do they equilibrate with the environment. In their normal terrestrial habitat evaporative losses can and do occur, and the animals appear to compensate for these in the laboratory by spending some time in the available water supply (personal observations; see also Gross, 1963). Thus we may conclude that the animals are capable of osmoregulation only to a limited extent (in part by behavioral mechanisms), and that the resulting fluctuations in serum concentrations are readily tolerated.

We wish to express our appreciation to the staff of the Lerner Marine Laboratory, Bimini, where this work was initiated; to Arnold Davidson for excellent technical assistance; to Dr. E. Bergofsky for performing some of the analyses and Dr. M. Mendelson for performing some of the tests on the efficacy of the Ringer. This work was supported by USPHS grant #AM 06268 to one of us (DMS) and by ONR assistance which made the preliminary work in the field possible.

Summary

1. From determinations of the principal electrolytes and respiratory gases in the serum of the land crab, *Gecarcinus lateralis,* a Ringer's solution has been devised and found to be effective in supporting neuromuscular activity for at least four hours in isolated preparations.

2. The animal is capable of a limited osmoregulation.

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